

EFFECTS OF $\text{NH}_4^+\text{-N}/\text{NO}_3^-\text{-N}$ RATIOS ON PHOTOSYNTHETIC CHARACTERISTICS, DRY MATTER YIELD AND NITRATE CONCENTRATION OF SPINACH

By SUZHI XING[†], JIANFEI WANG[†], YI ZHOU[†], SEAN A. BLOSZIES[‡],
CONG TU^{‡§} and SHUIJIN HU[‡]

[†]*College of Urban Construction & Environmental Sciences, Anhui Science and Technological University, Fengyang, Anhui 233100, P. R. China and* [‡]*Laboratory of Soil Ecology, Department of Plant Pathology, North Carolina State University, Raleigh, NC 27695-7616, USA*

(Accepted 27 June 2014; First published online 8 August 2014)

SUMMARY

Most plants prefer nitrate ($\text{NO}_3^-\text{-N}$) to ammonium ($\text{NH}_4^+\text{-N}$). However, high $\text{NO}_3^-\text{-N}$ in soil and water systems is a cause of concern for human health and the environment. Replacing $\text{NO}_3^-\text{-N}$ in plant nutrition regimes with an appropriate amount of $\text{NH}_4^+\text{-N}$ may alleviate these concerns. The purpose of this study was to evaluate the effects of different $\text{NH}_4^+\text{-N}/\text{NO}_3^-\text{-N}$ ratios on chlorophyll content, stomatal conductance, Rubisco activity, net photosynthetic rate, dry matter yield and $\text{NO}_3^-\text{-N}$ accumulation in spinach grown hydroponically. The $\text{NH}_4^+\text{-N}/\text{NO}_3^-\text{-N}$ percentage ratios were 0:100 (control), 25:75, 50:50, 75:25 and 100:0. Chlorophyll a and b, total chlorophyll, stomatal conductance, initial activity and activation state of Rubisco and net photosynthetic rate in spinach leaves were all reduced by increased $\text{NH}_4^+\text{-N}/\text{NO}_3^-\text{-N}$ ratios. Significant correlation existed between these measurements. However, no statistical differences in dry matter yield were revealed between the 0:100 and 25:75 treatments. Leaf nitrate concentrations were reduced by 38% at the 25:75 treatment relative to the 0:100 treatment. These findings suggest that lowering the relative proportion of $\text{NO}_3^-\text{-N}$ in fertilizer could effectively reduce $\text{NO}_3^-\text{-N}$ contents in leafy vegetables without decreasing their yields.

INTRODUCTION

Nitrogen (N) is an essential nutrient for plant growth. Plants can take up both ammonium ($\text{NH}_4^+\text{-N}$) and nitrate ($\text{NO}_3^-\text{-N}$) as their N source (Marschner, 1995). A multitude of studies have shown that most plants prefer $\text{NO}_3^-\text{-N}$ to $\text{NH}_4^+\text{-N}$ (Britto and Kronzucker, 2002; Marschner, 1995). With the exception of wetland species such as rice, plants supplied mainly with $\text{NO}_3^-\text{-N}$ typically grow larger and senesce later, while plants supplied with high $\text{NH}_4^+\text{-N}$ develop smaller leaves, grow smaller and yield less (Britto and Kronzucker, 2002; Gerendás *et al.*, 1997; Marschner, 1995).

Plant growth and productivity depend on photosynthesis. Plant photosynthesis is profoundly influenced not only by external factors, including light, temperature and CO_2 concentration, but also by internal forces such as chlorophyll content, enzyme activity and stomatal conductance (Hopkins and Hüner, 2009). As we have known, chlorophyll absorbs sunlight and uses its energy for plant photosynthesis, and

§Corresponding author. Email: cong_tu@ncsu.edu

ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) is a crucial enzyme involved in the fixation of CO₂ in photosynthesis. Nitrogen is an important constituent of chlorophyll as well as enzymes and proteins involved in photosynthesis. Up to 70–80% of the total leaf N is present in chloroplasts (Makino and Osmond, 1991), with 20 to 30% of the total leaf N contained within the protein Rubisco (Evans and Seemann, 1989; Kumar *et al.*, 2002; Makino, 2003). Thus, N limitation often leads to lowered Rubisco activity and photosynthesis (Evans, 1989). Accordingly, photosynthesis is positively and strongly dependent on N supply (Cechin and de Fátima Fumis, 2004; Evans, 1989; Pasquini and Santiago, 2012; Toth *et al.*, 2002). Moreover, plant photosynthesis has been shown to be higher when supplied with NO₃[−]-N instead of NH₄⁺-N (Guo *et al.*, 2006; Valentine *et al.*, 2002). Plant stomatal movements control gas (CO₂ and oxygen) exchange and transpiration rates, thus influencing photosynthesis. Research has shown that the presence of NH₄⁺-N reduces the stomatal conductance as compared with NO₃[−]-N (Hawkins and Lewis, 1993). In addition, it has also been demonstrated that a nitrate transporter (*atNRT1.1*) functions in stomatal opening (Guo *et al.*, 2003). Although the detrimental effects of NH₄⁺-N on plant growth, chlorophyll and photosynthesis have been documented (Britto and Kronzucker, 2002; Britto *et al.*, 2001; Gerendás *et al.*, 1997), recent research has revealed that NH₄⁺-N at low concentrations can be beneficial to plants (Azarmi and Esmailpour, 2010; Kotsiras *et al.*, 2002; Tabatabaei *et al.*, 2008). For example, Azarmi and Esmailpour (2010) observed that cucumber grown in a 25:75 NH₄⁺-N/NO₃[−]-N solution had the highest total fruit yield. Strawberry has also been found to produce the highest vegetative growth and fruit yield even at 50:50 NH₄⁺-N/NO₃[−]-N (Tabatabaei *et al.*, 2008). These studies imply that different plant species may have different optimal NH₄⁺-N/NO₃[−]-N ratios.

Applying NO₃[−]-N-form fertilizers often increases vegetable plant yield, but it may cause NO₃[−] accumulation within the plant, especially in leafy vegetables, which may be detrimental to human and animal health (Santamaria, 2006; Umar and Iqbal, 2007). Such applications may also lead to water pollution (Burow *et al.*, 2010) and increased trace gas emissions (van Groenigen *et al.*, 2010). Several studies have shown that combining NO₃[−]-N with small amounts of NH₄⁺-N as a N source can increase plant growth and dry biomass (Azarmi and Esmailpour, 2010; Claussen, 2002; Tabatabaei *et al.*, 2006, 2008) and decrease NO₃[−]-N in leaf tissues (Kotsiras *et al.*, 2002; Wang *et al.*, 2009).

Therefore, the objective of this study was to examine the influence of different NH₄⁺-N/NO₃[−]-N ratios on chlorophyll content, stomatal conductance, Rubisco activity and net photosynthetic rate, dry biomass and NO₃[−]-N accumulation in spinach grown hydroponically.

MATERIALS AND METHODS

Experiment

Spinach (*Spinacia oleracea* L., cv. Yinchuandayuan) seeds were surface-sterilized with 50 °C water for 30 min, washed thoroughly and soaked in deionized water for 12 h,

then sown in a nursery bed prepared with clean moist vermiculite in a greenhouse under natural photoperiod and day/night temperatures of 25 °C/15 °C. Thirty days after sowing, 30 seedlings of uniform size and vigour with four leaves were transplanted to each container and allowed to grow.

Five combinations of NH_4^+ -N/ NO_3^- -N percentage N ratios were used: 0:100 (control), 25:75, 50:50, 75:25 and 100:0. There were three replicates for each treatment. Complete randomized arrangement was used. Each container was given 12 L of full strength Hoagland solution. The concentrations of nutrients in the solution were 12 mmol N L^{-1} , 1 mmol P L^{-1} , 6 mmol K L^{-1} , 5 mmol Ca L^{-1} , 2 mmol Mg L^{-1} , 0.50 mg B L^{-1} , 2.8 mg Fe L^{-1} , 0.50 mg Mn L^{-1} , 0.05 mg Zn L^{-1} , 0.02 mg Cu L^{-1} and 0.09 mg Mo L^{-1} . A nitrification inhibitor, $\text{C}_2\text{H}_4\text{N}_4$ (dicyandiamide, 7 $\mu\text{mol L}^{-1}$), was added to each container to prevent nitrification. During the experiment, the solution was aerated for 30 min twice per day with an electronic inflator, and renewed every 10 days. The pH of the solution was monitored daily and kept at 6.50 by adding sufficient 0.5 mol L^{-1} HCl or NaOH.

Sampling and harvest

On the 36th day after transplanting, net photosynthetic rate and stomatal conductance of spinach leaves were measured *in situ*, and five measured plants were immediately removed from each container, rinsed with distilled water, blotted with paper towels and then divided into roots and aboveground part (consisting of leaf blades and petioles). Half of the leaves were used for chlorophyll extraction and the other half was kept at -80 °C for the Rubisco activity determination. To measure spinach biomass, another five plants were harvested from each container following the above cleaning and separation processes, dried at 65 °C and weighed.

Measurements

Chlorophyll content:. According to the method described by Ritchie (2006), 0.5 g of fresh spinach leaves (ground to <2 mm) were put into a 50 mL Erlenmeyer flask containing 20 mL of extraction solvent (ethanol:acetone:water = 4.5:4.5:1). The flask was sealed airtight and placed in dark for 24 h. One milliliter of the extracts was placed in a clean tube, and 4 mL fresh extraction solvent was then added. After thorough shaking, the absorbance was recorded at 645 nm and 663 nm on a spectrophotometer (SPECTRUM 756 UV, Shanghai Spectral Instrument Co., Shanghai, China). Chlorophyll concentrations in the extraction solution were then estimated using the Arnon equations (Arnon, 1949), and the leaf chlorophyll content was calculated according to the relative dilution quotient.

Rubisco activity:. The Rubisco activity was determined with a method modified from the method of Cheng and Fuchigami (2000). Samples (about 2.0 g each) stored at -80 °C were immediately homogenized with Rubisco extractant on an ice bath, filtered through four layers of gauze and centrifuged for 10 min at 15,000 \times g. The

supernatant was collected as a crude enzyme extract and frozen at 0 °C until further use.

The crude enzyme extract of 50 μL was pipetted into a semi-micro cuvette with 900 μL Rubisco determination solution and homogenized. Using distilled water as a blank and setting the absorbance value to zero at 340 nm on a UV-2450PC spectrometer (Shimadzu, Japan), the absorbance values of the samples were recorded for 0–40 s after addition of 50- μL 5-mmol L^{-1} RuBP. The initial Rubisco activity was calculated using the absolute descending value of the absorbance from 0 to 40 s.

The total Rubisco activity was measured as follows: The crude enzyme extracts and enzyme determination solution were kept at 30 °C for heat preservation and activation for 15 min before mixing with 50- μL 5-mmol L^{-1} RuBP. The subsequent steps were the same as the measurement of the initial Rubisco activity.

The Rubisco activation state refers to the ratio of the initial Rubisco activity to the total Rubisco activity (Cheng and Fuchigami, 2000).

Net photosynthetic rate and stomatal conductance: Net photosynthetic rate and stomatal conductance of the uppermost, fully expanded leaves were measured using a Li-Cor 6400 (LiCor, Lincoln, NE, USA). The leaf chamber was attached to one leaf for measurement at ambient CO_2 (380 ± 10 mmol CO_2 mol $^{-1}$ air, PPFD of 1500 mmol $\text{m}^{-2}\cdot\text{s}^{-1}$); leaf temperature was controlled at 25 ± 3 °C, and relative humidity in the leaf chamber was roughly 45% throughout the measurements.

Plant NO_3^- -N: Fresh samples (2.0 g) of the aboveground spinach were ground in 20-mL deionized water in an agate mortar, decolorized with activated carbon and then filtered to prevent chlorophyll pigment interference. The filtrate was used to measure NO_3^- -N concentration on a continuous-flow autoanalyser (Autoanalyzer 3, Bran + Luebbe GmbH, Germany; Wang *et al.*, 2009).

Statistical analysis

Experimental data were statistically analysed with single factor (ratios of NH_4^+ -N/ NO_3^- -N) analysis of variance (ANOVA) for completely randomized design using Statistical Analysis Software (SAS) version 9.1 for Windows. The least significant difference (LSD) method was used to separate the treatment means.

RESULTS

Chlorophyll content

Spinach leaf chlorophyll content differed significantly ($p < 0.05$) among treatments (Table 1). Chlorophyll a, chlorophyll b and total chlorophyll in spinach leaves decreased with increasing NH_4^+ -N/ NO_3^- -N ratios. A positive correlation existed between NO_3^- -N in the solution and chlorophyll a ($\text{Chl}_a = 0.6120 + 0.0770x$, $r = 0.9767$; $p < 0.01$), chlorophyll b ($\text{Chl}_b = 0.2060 + 0.0307x$, $r = 0.9830$; $p < 0.01$) and total chlorophyll ($\text{Chl}_t = 0.8160 + 0.1080x$, $r = 0.9797$; $p < 0.01$). Compared with the control (0:100), chlorophyll a, chlorophyll b and total chlorophyll from the

Table 1. Chlorophyll content in spinach leaves at different $\text{NH}_4^+ \text{-N}/\text{NO}_3^- \text{-N}$ ratios.

$\text{NH}_4^+ \text{-N}/\text{NO}_3^- \text{-N}$ ratio	Chlorophyll content (mg g^{-1} FW)			Chlorophyll a/ chlorophyll b ratio
	Chlorophyll a	Chlorophyll b	Total chlorophyll	
0:100	1.61 \pm 0.19 a	0.58 \pm 0.02 a	2.19 \pm 0.21 a	2.75 \pm 0.22 bc
25:75	1.20 \pm 0.06 b	0.48 \pm 0.03 b	1.68 \pm 0.07 b	2.51 \pm 0.15 c
50:50	1.04 \pm 0.03 bc	0.36 \pm 0.03 c	1.40 \pm 0.06 bc	2.89 \pm 0.13 b
75:25	0.93 \pm 0.06 c	0.34 \pm 0.03 c	1.27 \pm 0.09 c	2.77 \pm 0.12 bc
100:0	0.59 \pm 0.21 d	0.19 \pm 0.08 d	0.78 \pm 0.29 d	3.23 \pm 0.30 a
LSD _{0.05}	0.21	0.08	0.28	0.33

Values within a column followed by the same letter do not differ significantly at $p < 0.05$. Mean \pm SE of mean values ($n = 3$).

Table 2. Net photosynthetic rate and stomatal conductance of spinach leaves at different $\text{NH}_4^+ \text{-N}/\text{NO}_3^- \text{-N}$ ratio treatments.

$\text{NH}_4^+ \text{-N}/\text{NO}_3^- \text{-N}$ ratio	Stomatal conductance ($\text{mmol m}^{-2} \text{s}^{-1}$)	Net photosynthetic rate ($\text{CO}_2 \mu\text{mol m}^{-2} \text{s}^{-1}$)
0:100	273.0 \pm 15.7 a	24.0 \pm 1.54 a
25:75	264.6 \pm 12.0 a	19.7 \pm 2.39 b
50:50	249.6 \pm 11.2 ab	10.6 \pm 1.05 c
75:25	245.3 \pm 13.1 ab	6.56 \pm 0.91 d
100:0	223.4 \pm 17.3 b	3.88 \pm 1.57 d
LSD _{0.05}	29.4	2.80

Values within a column followed by the same letter do not differ significantly at $p < 0.05$. Mean \pm SE of mean values ($n = 3$).

25:75 treatment significantly decreased by 25.5%, 17.2% and 23.3% respectively. The chlorophyll a/chlorophyll b ratio showed little difference among the treatments except in the 100:0 treatment, which had a significantly higher chlorophyll a/chlorophyll b ratio than others.

Stomatal conductance

Stomatal conductance declined as $\text{NH}_4^+ \text{-N}/\text{NO}_3^- \text{-N}$ ratios increased (Table 2). Conductance was 3.1, 9.6, 10.1, and 18.2% lower in the 25:75, 50:50, 75:25 and 100:0 treatments, respectively, than in the control. However, there were no differences among the 0:100, 25:75 or 50:50 $\text{NH}_4^+ \text{-N}/\text{NO}_3^- \text{-N}$ ratio treatments.

Rubisco activity

The initial Rubisco activity dropped with increasing $\text{NH}_4^+ \text{-N}/\text{NO}_3^- \text{-N}$ ratios and significantly differed ($p < 0.05$) from the control (0:100) in the 50:50, 75:25 and 100:0 treatments (Figure 1A). However, there were no significant differences in the total Rubisco activity among the treatments. The Rubisco activation state was only significantly lower than the control (0:100) in the 75:25 and 100:0 treatments (Figure 1B).

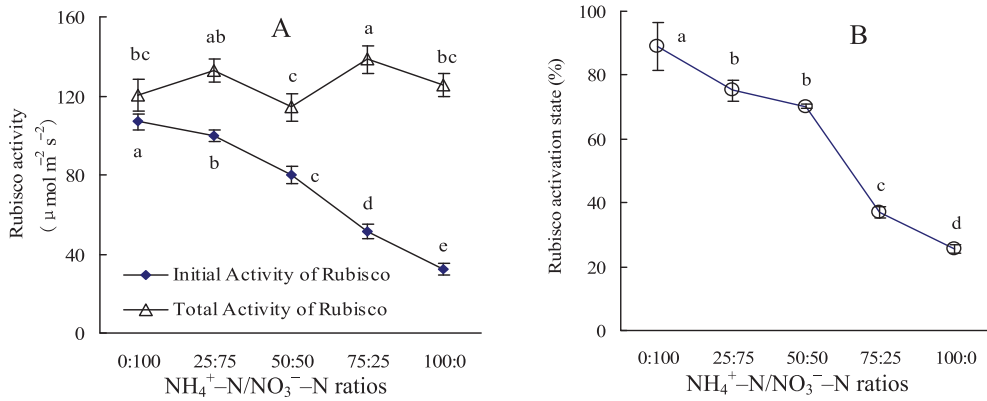


Figure 1. (a) Rubisco activity, and (b) activation state in spinach leaves under different $\text{NH}_4^+\text{-N}/\text{NO}_3^-\text{-N}$ ratios. The bars represent \pm SE of mean values. Data points with different letters indicate significant difference at $p < 0.05$.

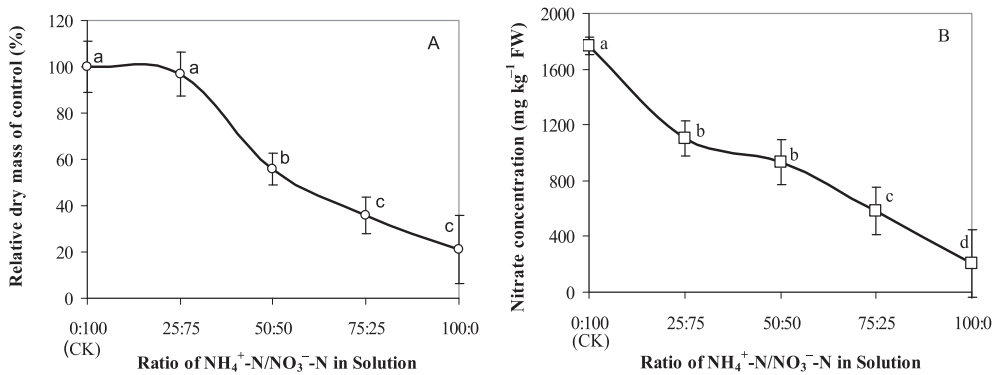


Figure 2. (a) Relative dry biomass, and (b) nitrate concentration of spinach as influenced by $\text{NH}_4^+\text{-N}/\text{NO}_3^-\text{-N}$ ratios in hydroponic culture. The bars represent \pm SE of mean values. Data points with different letters indicate significant difference at $p < 0.05$.

Net photosynthetic rate

The net photosynthetic rate of spinach leaves was reduced in the presence of $\text{NH}_4^+\text{-N}$ (Table 2). It was 82.1, 44.1, 27.3 and 16.2% of the control for the 25:75, 50:50, 75:25 and 100:0 treatments respectively. Significant differences in the net photosynthetic rate were found among all $\text{NH}_4^+\text{-N}/\text{NO}_3^-\text{-N}$ ratio treatments.

Dry matter yield and $\text{NO}_3^-\text{-N}$ accumulation

Spinach dry matter yield decreased as $\text{NH}_4^+\text{-N}/\text{NO}_3^-\text{-N}$ ratios increased (Figure 2A). However, there was no significant difference in the aboveground dry weights between 0:100 and 25:75 treatments. Dry matter yield of spinach had higher correlation with net photosynthetic rate and the initial Rubisco activity (Table 3).

Plant nitrate concentrations decreased with increasing $\text{NH}_4^+\text{-N}/\text{NO}_3^-\text{-N}$ ratios (Figure 2B). Biomass $\text{NO}_3^-\text{-N}$ concentrations were significantly positively correlated

Table 3. Correlation coefficients between dry matter yield (DMY), nitrate content (NIT), chlorophyll content (CHL), stomatal conductance (SC), initial Rubisco activity (IRA), Rubisco activation state (RAS) and net photosynthetic rate (NPR) of spinach leaves.

	DMY	NIT	CHL	SC	IRA	RAS
NIT	0.918					
CHL	0.926	0.990				
SC	0.954	0.953	0.978			
IRA	0.976	0.940	0.937	0.965		
RAS	0.937	0.949	0.924	0.930	0.986	
NPR	0.987	0.951	0.953	0.949	0.956	0.928

Critical values for correlation coefficient significance are 0.878 at $p = 0.05$ and 0.959 at $p = 0.01$. $n = 5$.

with the proportion of the total N supplied as NO_3^- -N ($r = 0.983^{**}$), chlorophyll contents, stomatal conductance and other measurements (Table 3). Plant NO_3^- -N concentration was reduced by 38% at the 25:75 treatment in comparison with the 0:100 treatment.

DISCUSSION

Our experimental data showed that higher proportions of NH_4^+ -N in the N supply to spinach significantly reduced the chlorophyll content, stomatal conductance, Rubisco activity and net photosynthetic rate and thus dry matter yield (Table 3), but no statistical differences in dry matter yield were found between the 0:100 and 25:75 NH_4^+ -N/ NO_3^- -N ratios, implying that increasing the NO_3^- -N proportion of the total N above 75% does not result in higher biomass accumulation. Previous reports have shown that plants supplied with NH_4^+ -N as the sole or major N source develop smaller leaves (Walch-Liu *et al.*, 2000) and grow more slowly (Claussen and Lenz, 1999; Guo *et al.*, 2007). Conversely, increased plant growth and yield have been reported when plants were fertilized mainly with NO_3^- -N (25:75 NH_4^+ -N to NO_3^- -N; Azarmi and Esmacilpour, 2010; Chen *et al.*, 2009; Tabatabaei *et al.*, 2006). Several causes and/or mechanisms have been proposed to explain ammonium's (NH_4^+) toxicity to plants. For instance, NH_4^+ reduces cell division and elongation (Walch-Liu *et al.*, 2000), resulting in smaller plants. High external NH_4^+ may break down the regulation of its influx into root cells, leading to an elevated efflux of the influxed NH_4^+ , a 40% increase in energetic cost of root cells, and thus to a decline in growth (Britto *et al.*, 2001). It has been also demonstrated that NH_4^+ at higher concentrations can uncouple electron transport from photophosphorylation, thus reducing photosynthetic rates (Peltier and Thibault, 1983). The presence of a dual-affinity nitrate transporter gene (atNRT1.1) in guard cells has demonstrated an important role of NO_3^- -N in regulating stomatal opening and thus photosynthesis and growth (Guo *et al.*, 2003). We infer that lowered photosynthesis at high levels of NH_4^+ -N could also be due to a reduction in light absorption resulting from lower chlorophyll concentrations (Table 1), smaller leaf size (data not shown) and reduced stomatal conductance (Table 2). Indeed, high NH_4^+ -N has been shown to reduce stomatal conductance (Hawkins and Lewis,

1993; Lopes and Araus, 2006) as well as to suppress plant uptake of potassium (K^+), an important regulator of stomatal movement (Outlaw Jr., 1983; Wang *et al.*, 2003). The combination of both elevated NH_4^+-N supply and diminished NO_3^--N reduced photosynthesis and yield.

The current results showed no statistical differences in dry matter yield between the 0:100 and 25:75 NH_4^+-N/NO_3^--N ratios. In a preliminary experiment with different concentrations of NO_3^--N alone, it was found that spinach biomass significantly declined (18% less) when NO_3^--N concentration was lowered from 12 mM to 9 mM. Therefore, replacing this deficit in NO_3^--N with small amounts of NH_4^+-N can actually maintain plant biomass. In combination, our plant biomass and net photosynthetic rate results also imply that dark respiration under NH_4^+-N nutrition might be more efficient than under NO_3^--N nutrition because plant dry matter accumulation is the balance between photosynthetic gains and respiratory losses. Bloom *et al.* (1992) found that barley used 23% of root carbon catabolism for N absorption and assimilation under NO_3^--N nutrition and only 14% under NH_4^+ nutrition. Barker *et al.* (1965) also observed that excised bean leaves assimilated an average of 46% of the absorbed NH_4^+ into organic compounds, compared with 28% of the absorbed NO_3^- . Therefore, plants supplied with NH_4^+ might increase the efficiency of carbon catabolism, although NH_4^+ usually inhibited plant photosynthesis. Thus, plant dry biomass may not necessarily decline under low NH_4^+ nutrition.

Our study also showed that partial replacement of NO_3^--N with NH_4^+-N markedly reduced the NO_3^--N content in spinach without significantly decreasing biomass at the 25:75 NH_4^+-N/NO_3^--N ratio. This may be pertinent to both vegetable production and environmental quality concerns. Although NO_3^--N is naturally present in soils, water and plants, excess NO_3^--N in plants (particularly in leafy vegetables) may harm human health (Chan, 2011; Santamaria, 2006). To protect consumers, several countries have already established maximum NO_3^--N levels in vegetables (Santamaria, 2006). Measures to reduce the NO_3^--N content in vegetable plants have been proposed, including genotype selection, balanced fertilization, altered nitrogen forms and organic farming (Umar and Iqbal, 2007). The current experiment highlighted that applying 25% of N nutrition as NH_4^+-N could reduce NO_3^--N concentrations in spinach by 38% without influencing spinach biomass production (Figure 2). Furthermore, reduction in NO_3^--N fertilizer usage may lower soil NO_3^- levels, thereby mitigating not only water contamination of NO_3^--N via leaching (Burow *et al.*, 2010) but also N_2O emission to the atmosphere via denitrification (Gollany *et al.*, 2004; Goulding, 2000). However, NH_4^+-N fertilizer should be applied in combination with a nitrification inhibitor as NH_4^+ can quickly convert into NO_3^- (Di *et al.*, 2009).

In conclusion, higher NH_4^+-N/NO_3^--N ratios decreased the chlorophyll content, net photosynthetic rate, stomatal conductance and the Rubisco activity of spinach leaves. However, no statistical differences in plant biomass were found between the 0:100 and 25:75 NH_4^+-N/NO_3^--N ratios. Nitrate concentrations in spinach leaves were reduced by 38% at the 25:75 NH_4^+-N/NO_3^--N treatment compared with the 0:100 treatment. These findings suggest that an appropriate combination of NO_3^--N

and NH_4^+ -N would not impact spinach yield while greatly reducing NO_3^- -N content in this leafy vegetable.

Acknowledgements. This study was supported in part by the Science-Technology Project of An' hui Province, China (No: 1101C0603046) and the Transformation Program in Agricultural Scientific and Technological Achievements of An' hui Province, China (No: 10140306017). The authors would like to thank the two anonymous reviewers for their valuable suggestions on improving the manuscript.

REFERENCES

- Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts – polyphenoloxidase in *Beta-vulgaris*. *Plant Physiology* 24:1–15.
- Azarmi, R. and Esmailpour, B. (2010). Effect of NO_3^- to NH_4^+ ratio on growth, yield and element composition of cucumber (*Cucumis sativus* L.). *Journal of Food, Agriculture & Environment* 8:607–610.
- Barker, A. V., Volk, R. J. and Jackson, W. A. (1965). Effects of ammonium and nitrate nutrition on dark respiration of excised bean leaves. *Crop Science* 5:439–444.
- Bloom, A. J., Sukrapanna, S. S. and Warner, R. L. (1992). Root respiration associated with ammonium and nitrate absorption and assimilation by barley. *Plant Physiology* 99:1294–1301.
- Britto, D. T. and Kronzucker, H. J. (2002). NH_4^+ toxicity in higher plants: a critical review. *Journal of Plant Physiology* 159:567–584.
- Britto, D. T., Siddiqi, M. Y., Glass, A. D. M. and Kronzucker, H. J. (2001). Futile transmembrane NH_4^+ cycling: a cellular hypothesis to explain ammonium toxicity in plants. *Proceedings of the National Academy of Sciences of the United States of America* 98:4255–4258.
- Burow, K. R., Nolan, B. T., Rupert, M. G. and Dubrovsky, N. M. (2010). Nitrate in groundwater of the United States, 1991–2003. *Environmental Science and Technology* 44:4988–4997.
- Cechin, I. and de Fátima Fumis, T. (2004). Effect of nitrogen supply on growth and photosynthesis of sunflower plants grown in the greenhouse. *Plant Science* 166:1379–1385.
- Chan, T. Y. K. (2011). Vegetable-borne nitrate and nitrite and the risk of methaemoglobinaemia. *Toxicology Letters* 200:107–108.
- Chen, L., Liu, S. C., Gai, J. Y., Zhu, Y. L., Yang, L. F. and Wei, G. P. (2009). Effects of nitrogen forms on the growth, ascorbate-glutathione cycle and lipid peroxidation in developing seeds of vegetable soybean. *African Journal of Agricultural Research* 4:1178–1188.
- Cheng, L. L. and Fuchigami, L. H. (2000). Rubisco activation state decreases with increasing nitrogen content in apple leaves. *Journal of Experimental Botany* 51:1687–1694.
- Claussen, W. (2002). Growth, water use efficiency, and proline content of hydroponically grown tomato plants as affected by nitrogen source and nutrient concentration. *Plant and Soil* 2:199–209.
- Claussen, W. and Lenz, F. (1999). Effect of ammonium or nitrate nutrition on net photosynthesis, growth, and activity of the enzymes nitrate reductase and glutamine synthetase in blueberry, raspberry and strawberry. *Plant and Soil* 208:95–102.
- Di, H. J., Cameron, K. C., Shen, J. P., Winefield, C. S., O'Callaghan, M., Bowatte, S. and He, J. Z. (2009). Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. *Nature Geoscience* 2:621–624.
- Evans, J. R. (1989). Photosynthesis and nitrogen relationships in leaves of C_3 plants. *Oecologia* 78:9–19.
- Evans, J. R. and Seemann, J. R. (1989). The allocation of protein nitrogen in the photosynthetic apparatus: cost, consequences and control. In *Photosynthesis*, 183–205 (Ed W. R. Briggs). New York, NY: Alan R. Liss.
- Gerendás, J., Zhu, Z., Bendixen, R., Ratcliffe, R. G. and Sattelmacher, B. (1997). Physiological and biochemical processes related to ammonium toxicity in higher plants. *Zeitschrift Fur Pflanzenernahrung Und Bodenkunde* 160:239–251.
- Gollany, H. T., Molina, J.-A. E., Allmars, R. R., Layese, M. F., Baker, J. M. and Cheng, H. H. (2004). Nitrogen leaching and denitrification in continuous corn as related to residue management and nitrogen fertilization. *Environmental Management* 33:S289–S298.
- Goulding, K. (2000). Nitrate leaching from arable and horticultural land. *Soil Use and Management* 16:145–151.

- Guo, H. X., Liu, W. Q. and Shi, Y. C. (2006). Effects of different nitrogen forms on photosynthetic rate and the chlorophyll fluorescence induction kinetics of flue-cured tobacco. *Photosynthetica* 44:140–142.
- Guo, F.-Q., Young, L. and Crawford, N. M. (2003). The nitrate transporter *atNRT1.1* (CHL1) functions in stomatal opening and contributes to drought susceptibility in Arabidopsis. *The Plant Cell* 15:107–117.
- Guo, S., Zhou, Y., Shen, Q. and Zhang, F. (2007). Effect of ammonium and nitrate nutrition on some physiological processes in higher plants – growth, photosynthesis, photorespiration, and water relations. *Plant Biology* 9:21–29.
- Hawkins, H.-J. and Lewis, O. A. M. (1993). Combination effect of NaCl salinity, nitrogen form and calcium concentration on the growth, ionic content and gaseous exchange properties of *Triticum aestivum* L. cv. Gamtoos. *New Phytologist* 124:161–170.
- Hopkins, W. G. and Hüner, N. P. A. (2009). *Introduction to Plant Physiology*, 4th edn. Hoboken, NJ: John Wiley.
- Kotsiras, A., Olympios, C. M., Drosopoulos, J. and Passam, H. C. (2002). Effects of nitrogen form and concentration on the distribution of ions within cucumber fruits. *Scientia Horticulturae* 95:175–183.
- Kumar, P. A., Parry, M. A. J., Mitchell, R. A. C., Ahmad, A. and Abrol, Y. P. (2002). Photosynthesis and nitrogen use-efficiency. In *Photosynthetic Nitrogen Assimilation and Associated Carbon and Respiratory Metabolism*, 23–34 (Eds C. H. Foyer and G. Noctor). Dordrecht, Netherlands: Kluwer.
- Lopes, M. S. and Araus, J. L. (2006). Nitrogen source and water regime effects on durum wheat photosynthesis and stable carbon and nitrogen isotope composition. *Physiologia Plantarum* 126:435–445.
- Makino, A. (2003). Rubisco and nitrogen relationships in rice: leaf photosynthesis and plant growth. *Soil Science and Plant Nutrition* 49:319–327.
- Makino, A. and Osmond, B. (1991). Effects of nitrogen nutrition on nitrogen partitioning between chloroplasts and mitochondria in pea and wheat. *Plant Physiology* 96:355–362.
- Marschner, H. (1995). *Mineral Nutrition of Higher Plants*, 2nd edn. London Academic Press.
- Outlaw Jr., W. H. (1983). Current concepts on the role of potassium in stomatal movements. *Physiologia Plantarum* 59:302–311.
- Pasquini, S. C. and Santiago, L. S. (2012). Nutrients limit photosynthesis in seedlings of a lowland tropical forest tree species. *Oecologia* 168:311–319.
- Peltier, G. and Thibault, P. (1983). Ammonia exchange and photo-respiration in chlamydomonas. *Plant Physiology* 71:888–892.
- Ritchie, R. J. (2006). Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. *Photosynthesis Research* 89:27–41.
- Santamaria, P. (2006). Nitrate in vegetables: toxicity, content, intake and EC regulation. *Journal of the Science of Food and Agriculture* 86:10–17.
- Tabatabaei, S. J., Fatemi, L. S. and Fallahi, E. (2006). Effect of ammonium: nitrate ratio on yield, calcium concentration, and photosynthesis rate in strawberry. *Journal of Plant Nutrition* 29:1273–1285.
- Tabatabaei, S. J., Yusefi, M. and Hajiloo, J. (2008). Effects of shading and $\text{NO}_3\text{:NH}_4$ ratio on yield, quality and N metabolism in strawberry. *Scientia Horticulturae* 116:264–272.
- Toth, V. R., Meszaros, I., Veres, S. and Nagy, J. (2002). Effects of the available nitrogen on the photosynthetic activity and xanthophyll cycle pool of maize in field. *Journal of Plant Physiology* 159:627–634.
- Umar, A. S. and Iqbal, M. (2007). Nitrate accumulation in plants, factors affecting the process, and human health implications. A review. *Agronomy for Sustainable Development* 27:45–57.
- Valentine, A. J., Osborne, B. A. and Mitchell, D. T. (2002). Form of inorganic nitrogen influences mycorrhizal colonisation and photosynthesis of cucumber. *Scientia Horticulturae* 92:229–239.
- van Groenigen, J. W., Velthof, G. L., Oenema, O., van Groenigen, K. J. and van Kessel, C. (2010). Towards an agronomic assessment of N_2O emissions: a case study for arable crops. *European Journal of Soil Science* 61:903–913.
- Walch-Liu, P., Neumann, G., Bangerth, F. and Engels, C. (2000). Rapid effects of nitrogen form on leaf morphogenesis in tobacco. *Journal of Experimental Botany* 51:227–237.
- Wang, G. Y., Li, C. J. and Zhang, F. S. (2003). Effects of different nitrogen forms and combination with foliar spraying with 6-benzylaminopurine on growth, transpiration, and water and potassium uptake and flow in tobacco. *Plant and Soil* 256:169–178.
- Wang, J., Zhou, Y., Dong, C., Shen, Q. and Putheti, R. (2009). Effects of $\text{NH}_4^+\text{-N/NO}_3^-\text{-N}$ ratios on growth, nitrate uptake and organic acid levels of spinach (*Spinacia oleracea* L.). *African Journal of Biotechnology* 8:3597–3602.